

NATIVE AND ENGINEERED SIMULANTS FOR DNA VIRUS THREAT AGENTS

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1. INTRODUCTION

The ability to test BW detection gear in all stages of development (lab bench, test chamber, and field) and to train using the gear in realistic situations is a cornerstone of biological defense research and operations. Better simulants will allow higher quality and more reproducible testing, as well as more realism in testing and training.

There is currently only one virus widely accepted for low-level containment or field release in the study of biological detection, the bacteriophage MS2. While MS2 has been used for decades in the bio-defense research, development, testing, and evaluation communities in lieu of actual viral threat agents, MS2 is less than ideal for several reasons. It is not the same size as most of the recognized threat agents. It does not have the same capsid structure as many viruses of eukaryotes, and the size of the capsid is considerably smaller than many animal viruses. In particular, it is not similar in nearly every respect to any of the orthopoxviruses, a family that includes viral agents of concern. Poxviruses have a virion particle size 20-40 times longer and wider than MS2, has both enveloped and non-enveloped forms (whereas MS2 has only a single virion particle form), and has a double-stranded DNA genome (while the MS2 genome is single-stranded RNA).

There is a clear demand from the bio-defense community for a virus to use in place of MS2 as a simulant that more closely resembles some viruses of mammals. We are currently examining alternatives to MS2 among the DNA viruses, with desirable traits including lack of mammalian pathogenicity, similarity in size and composition to poxviruses, acceptability to regulatory agencies that must approve plans for environmental test releases, and the potential to engineer the structure or genome sequence of the candidate simulant viruses to provide unique DNA or epitope tags for identification.

Our current candidate of choice is a virus from the baculovirus family. Baculoviruses possess many of the properties outlined above. They are double-stranded DNA (dsDNA) viruses that do not infect mammals. They are closer in size to poxviruses than MS2 is (150-250 nm vs. 26 nm) (Fig. 1). They are well characterized at the molecular level, and have served in the laboratory setting as a vehicle for the abundant expression of recombinant

proteins (O'Reilly et al. 1994, Anderson et al. 2002). Perhaps most importantly, they have been released to the environment in both native and engineered forms (Mulock and Falkner 1997), suggesting that regulatory obstacles to their use in the field may not be insurmountable. Lastly, baculoviruses have been engineered so that they display peptides and proteins on the virion surface (Ernst et al. 2002, Yoshida et al. 2003), demonstrating the feasibility of modifying baculoviruses to display epitopes which mimic threat agents in testing and evaluation settings without conferring pathogenicity.

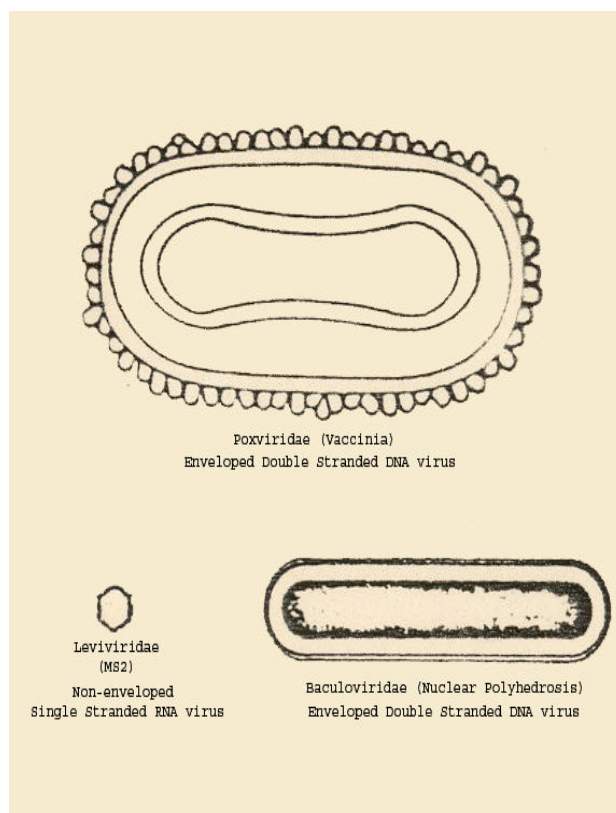


Figure 1. Line drawings of (clockwise from top) vaccinia virus, a baculovirus (nuclear polyhedrosis virus) and bacteriophage MS2, approximately to scale. Note that the baculovirus more closely resembles vaccinia in shape, size, presence of envelope, and genome composition than does MS2.

| Report Documentation Page | | | | Form Approved OMB No. 0704-0188 | |
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| 1. REPORT DATE 00 DEC 2004 | | 2. REPORT TYPE N/A | | 3. DATES COVERED - | |
| 4. TITLE AND SUBTITLE Native And Engineered Simulants For Dna Virus Threat Agents | | | | 5a. CONTRACT NUMBER g | |
| | | | | 5b. GRANT NUMBER | |
| | | | | 5c. PROGRAM ELEMENT NUMBER | |
| 6. AUTHOR(S) | | | | 5d. PROJECT NUMBER | |
| | | | | 5e. TASK NUMBER | |
| | | | | 5f. WORK UNIT NUMBER | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) U. S. Army Edgewood Chemical Biological Center Research, Development, and Engineering Command Aberdeen Proving Ground, Maryland 21010 USA | | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) | | | | 10. SPONSOR/MONITOR'S ACRONYM(S) | |
| | | | | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) | |
| 12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited | | | | | |
| 13. SUPPLEMENTARY NOTES See also ADM001736, Proceedings for the Army Science Conference (24th) Held on 29 November - 2 December 2005 in Orlando, Florida., The original document contains color images. | | | | | |
| 14. ABSTRACT | | | | | |
| 15. SUBJECT TERMS | | | | | |
| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT UU | 18. NUMBER OF PAGES 5 | 19a. NAME OF RESPONSIBLE PERSON |
| a. REPORT unclassified | b. ABSTRACT unclassified | c. THIS PAGE unclassified | | | |

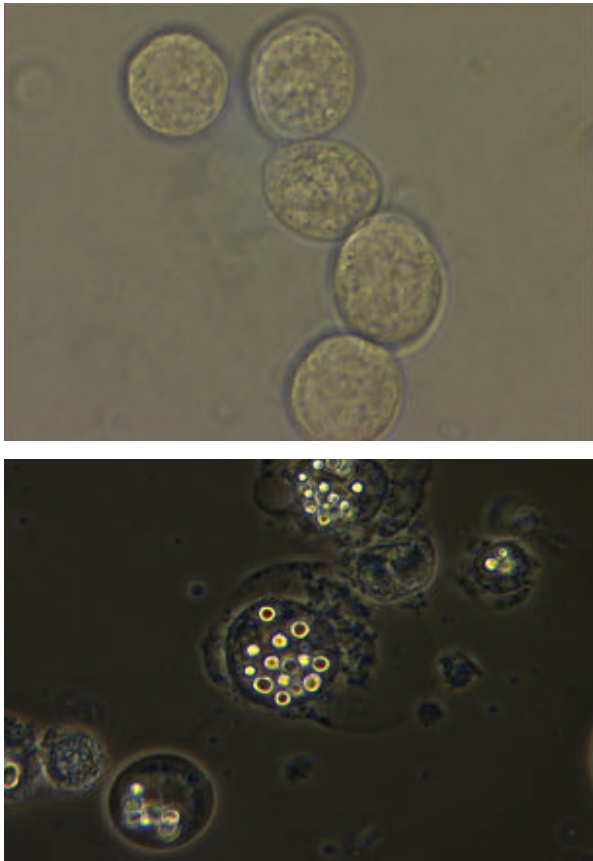


Figure 2. Light microscopy of AcMNPV-infected *Sf9* cells. Top Panel: cells infected with AcMNPV at an earlier stage, producing budded virus. Individual virions (budded virus) are too small to visualize under the light microscope. (magnification 400x). Bottom Panel: cells containing occlusion bodies visualized under phase contrast optics. Occlusion bodies consist of virions enmeshed in a matrix of polyhedrin protein, which obscures surface glycoproteins from recognition by antibodies.

Among the first tasks in the establishment of a new simulant must be the examination of protocols for producing, purifying, and detecting the presence of the simulant. In order to become broadly applicable and widely accepted, a simulant must not only adequately resemble the agent in key characteristics, but also be economically producible in a way that can be standardized. There must also be a suite of reagents and methods available for its detection under a variety of conditions. Therefore, our initial efforts have focused on protocols for the production and detection of baculovirus.

2. MATERIALS AND METHODS

Standard protocols for the cultivation of baculovirus (O'Reilly et al. 1994) were optimized to produce large quantities of baculovirus, *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) in cultured *Spodoptera frugiperda* (*Sf9*) cells. Virus was added to active *Sf9* cultures at a multiplicity of infection

(MOI) of 0.1, using the following formula: inoculum required (ml) = desired MOI x total number of cells / titer of viral inoculum (pfu/ml).

Purification of budded virus from a 72-hour culture of infected *Sf9* cells was carried out as a three-step process. In order to remove infected cells and cells debris cultures were centrifuged for 10 minutes at 1200 RPM. Supernatant was collected and further purified by centrifugation at 6000 RPM followed by filtration through a 0.22µm low-protein binding filter.

3.0 RESULTS

3.1 Growth, harvest and purification of virions.

Cells that are grown in serum free suspension cultures, at 28°C and 130 RPM, produced more viable cells as well as a greater yield of virions compared to cells grown in monolayers and not shaken (data not shown). Infected cultures incubated for 7 days yielded increased amounts of occluded virus (Fig. 2); these occlusion bodies contain virus, but are much larger than threat viruses; therefore, we have limited our efforts to examining virions (budded virus) that are not enmeshed in a polyhedrin matrix and are produced prior to the appearance of occluded virus. Because some simulant applications may require concentrated sample, we examined a number of methods of harvesting virions, including several combinations of low speed centrifugation and filtration, and two ultracentrifugation protocols using sucrose gradients and cushions. Centrifuging the sample through a sucrose cushion was effective and yielded virion pellets; however, the small sample size accommodated by the ultracentrifuge made this method impractical for large scale processes. The two-step centrifugation process, followed by filtration gave better yields with less effort, and may be scalable.

3.2 Determination of viral titer.

Plaque-forming units (pfu) were enumerated by two methods: a standard plaque assay using lawns of *Sf9* cells in 6-well culture dishes (O'Reilly et al. 1994), or using a commercially available baculovirus titrating kit (BacPak rapid titer kit, Clontech). Results obtained using each method were comparable; a concentrated viral preparation was found to contain 2×10^8 pfu/ml by both methods. The BacPak system is based on an enzyme-linked immunosorbent method (ELISA) that employs an antibody that recognizes the surface protein gp64, whereas the viral plaque assay (O'Reilly et al. 1994) determined titer indirectly by displaying the effect of virus on lawns of host (*Sf9*) cells. While the BacPak gave results in fewer days, the assay was more labor-intensive, and the cost per assay was prohibitive for constant use.

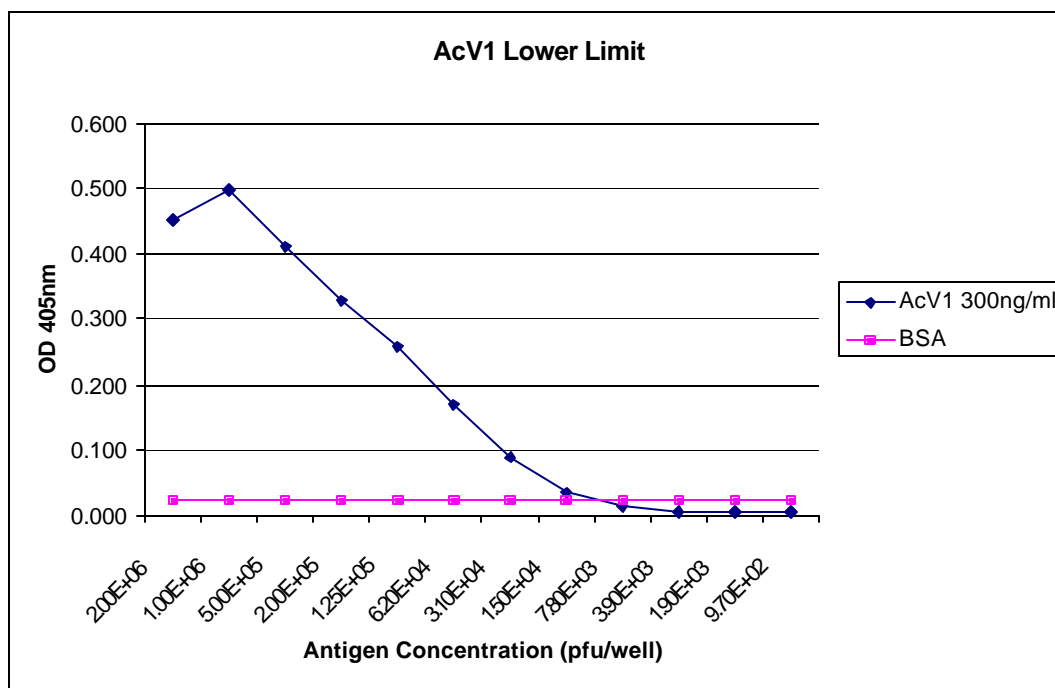


Figure 3 Detection of AcMNPV virions by direct Enzyme-Linked Immunosorbent Assay (ELISA). After allowing suspensions of virions to bind to the wells in the assay plate, a commercially available antibody (AcV1), which binds a protein on the surface of the virion, was added at a concentration of 300 ng/ml. The presence of bound AcV1 (and therefore virus) in each well was detected with a secondary antibody conjugated to the enzyme horseradish peroxidase and a chromogenic substrate with an absorbance maximum of approximately 405 nm.

3.3 Detection by immunoassay.

Before we undertook efforts to develop anti-AcMNPV antibodies, we purchased two commercial monoclonal antibodies (AcV1 and AcV5), both of which bind protein gp64 of the surface of budded virus particles (eBioscience, Inc.). Several dot blots were performed to determine initially whether AcV1 and AcV5 reacted with virus particles. Both antibodies reacted with supernatants from infected *Sf9* cultures (containing viral particles) sampled up to 72 hours; samples of supernatant collected after that time showed decreased signal with both antibodies (data not shown). This result is consistent with the binding of both antibodies to gp64, which is exposed on the surface of budded virus. However, gp64 is obscured by the polyhedrin matrix that surrounds virions in occlusion bodies formed later during infection (Fig.3). ELISAs were performed to assess the limit of detection for budded virus in culture supernatants. Preliminary data indicate that as few as 10^6 particles can be detected in a direct ELISA; however, we are continuing to optimize the antibody and antigen concentrations in this assay.

3.4 Detection by polymerase chain reaction(PCR).

We have designed probe and primer sequences comprising two real-time PCR ("TaqMan") assays, targeting sequences in the genes encoding the glycoproteins gp64 and Ac-Orf18 (designated gp18) of

AcMNPV. An analysis of the PCR product of the gp64 assay reveals the presence of an amplicon of the expected size (70base pairs, Figure 4). TaqMan PCR reactions using the primer sets for detecting gp18 and gp64 are still being tested.

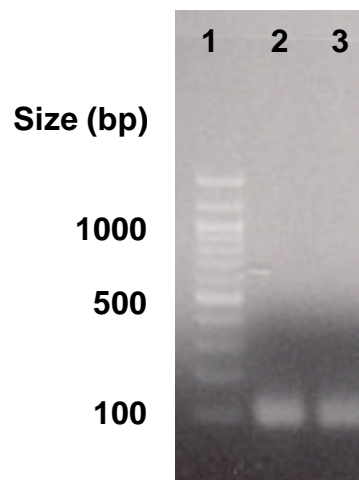


Figure 4. Agarose gel electrophoresis of DNA produced by the PCR assay for gp64. Lane 1, 100 base pair ladder for size comparison. Lanes 2 and 3, PCR product. Reactions included 9×10^5 virions.

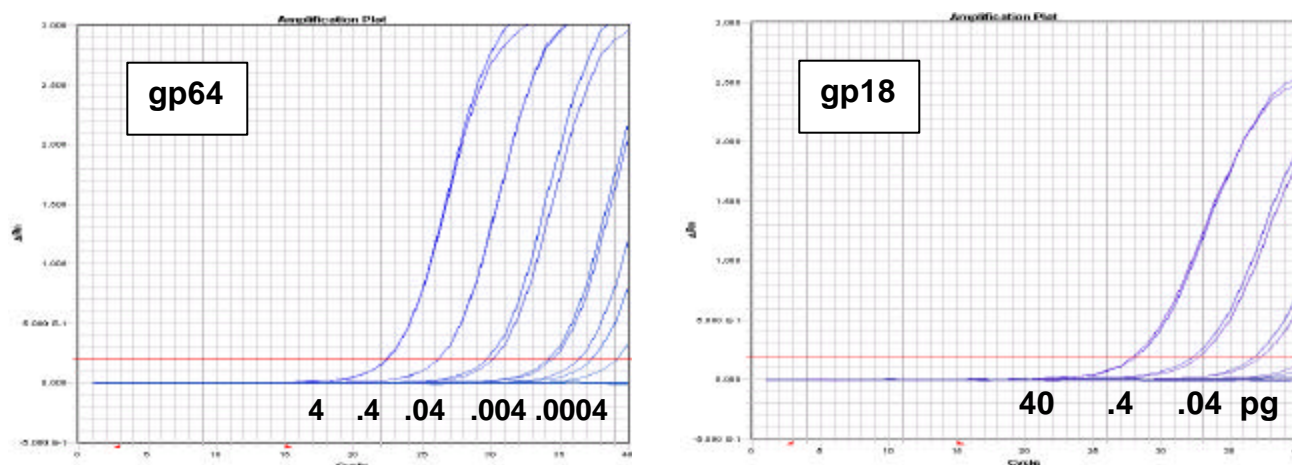


Figure 5. Detection of DNA sequences in purified AcMNPV DNA by real-time fluorogenic PCR using the DNA-binding dye SYBR Green. Numbers in bold indicate picograms of DNA detected. Left, detection of target sequence in the gp64 gene. The assay repeatably detected 400 ag of viral genomic DNA, approximately the mass of a single genome. Right, detection of target sequence in the gp18 gene. The gp18 assay was less sensitive, with a repeatable limit of detection of 40 fg.

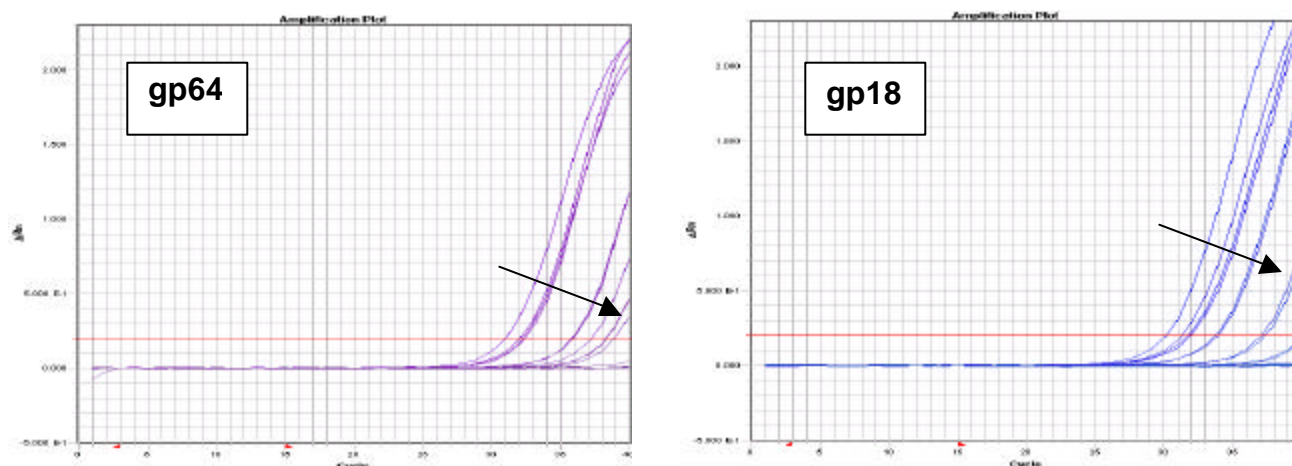


Figure . 6 Detection of AcMNPV virions by real-time fluorogenic PCR using the DNA-binding dye SYBR Green. Left, detection of virions using the target sequence in the gp64 gene. The assay repeatably detected about 4 virions (bold arrow). Right, detection AcMNPV virions using the target sequence in the gp18 gene. Again, the gp18 assay was less sensitive, with a repeatable limit of detection of about 40 virions. (bold arrow).

However, the primer sets provide sensitive real-time fluorogenic PCR detection of the target sequences when used with the DNA-binding dye SYBR Green (Figure 5). The assay we designed that targets gp64 has a limit of detection of as little as 400 attograms (ag) of viral DNA, a quantity equivalent to about a single copy of the viral genome. Limit-of-detection experiments on intact virions showed similar sensitivity (Figure 6). Lower levels of

virion detection in the gp18 experiments suggest that there may have been target DNA present in the form of denatured or otherwise non-viable virus particles. That is, PCR (and immunological assays as well) may overestimate the number of viable viruses in a preparation, as the proteins or nucleic acids of non-viable viruses can react with the PCR or antibody reagents in an assay.

3.5 Survival of virions

An important use for microbial simulants in the study of biological defense is their use in determining the effectiveness of decontaminating agents, and their use as models for the fate of BW agents dispersed in the environment. For that reason, it is important to understand the ability of microbial simulants to withstand environmental stress, and to assess their resistance or susceptibility relative to BW agents. To begin to characterize the ability of AcMNPV virions to withstand environmental stress, samples were subjected to heat, multiple freeze-thaw cycles, drying on surfaces, and lyophilization (freeze-drying).

CONCLUSIONS

Our results to date indicate that baculoviruses may be effective simulants for large dsDNA virus threat agents in the laboratory setting. We are continuing to refine methods for production and purification of baculovirus virions, as well as the detection of native, non-engineered AcMNPV by both immunological and nucleic acid-based methods. We are also beginning to examine the engineering of the AcMNPV genome to include short oligonucleotides that will serve as threat agent target sequences. Such engineered simulants will allow the possibility of using actual fielded BW detection reagents (in controlled settings) with a simulant, possibly eliminating the need for separate reagent sets for simulants. To extend this idea to immunologically detectable features on the virion surface, we are leveraging on-going efforts by other workers to develop peptide “mimetopes” that can be displayed on a baculovirus virion surface, to provide improved immunological simulation. Candidate synthetic peptides have been isolated that are recognized by anti-poxvirus antibodies (R. Thompson, unpublished data); these may serve as the first candidates for baculovirus surface display.

Future studies will focus on understanding the survival of AcMNPV under additional stress conditions in comparison to vaccinia virus, as well as constructing proof-of-principle engineered AcMNPV strains. Lastly, we are examining other viruses that may also serve as simulants for poxviruses, to expand the number of tools available to the biological defense RDT&E community.

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